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Purification and Structural Determination of SCB1, a γ-Butyrolactone That Elicits Antibiotic Production in *Streptomyces coelicolor* A3(2)*

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Early stationary phase culture supernatants of *Streptomyces coelicolor* A3(2) contained at least four small diffusible signaling molecules that could elicit precocious antibiotic synthesis in the producing strain. The compounds were not detected in exponentially growing cultures. One of these compounds, SCB1, was purified to homogeneity and shown to be a γ-butyrolactone of structure (2*R*,3*R*,1′*R*)-2-(1′-hydroxy-6-methylheptyl)-3-hydroxymethylbutanolide. Bioassays of chemically synthesized SCB1, and of its purified stereoisomers, suggest that SCB1 acts in a highly specific manner to elicit the production of both actinorhodin and undecylprodigiosin, the two pigmented antibiotics made by *S. coelicolor*.

Small diffusible signaling molecules play regulatory roles in a wide variety of bacteria. The most intensively studied are the *N*-acyl homoserine lactones, which have diverse roles as signaling molecules in a wide range of Gram-negative bacteria (1). Among Gram-positive bacteria, structurally similar but chemically distinct γ-butyrolactones play determining roles in antibiotic production and sporulation in *Streptomyces* species (2) (Fig. 1). Streptomycetes are mycelial soil bacteria with a developmental program that results in sporulation. They also produce a wide variety of antibiotics with important uses in medicine and in agriculture. These antibiotics are the products of complex biosynthetic pathways, activated typically in a growth phase-dependent manner (3). In liquid culture, antibiotic production generally occurs in stationary phase (4, 5), while in surface-grown cultures, it usually coincides with the onset of morphological differentiation, *i.e.* the formation of aerial hyphae.

The most intensively studied γ-butyrolactone is A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone), which is required for streptomycin production and sporulation in *Streptomyces griseus* (6). A-factor binds to a cytoplasmic protein that in its absence represses streptomycin production and morphological differentiation (7). Other γ-butyrolactones have also been shown to induce antibiotic biosynthesis, *e.g.* the virginiae butanolides of *Streptomyces virginiae* (8, 9). Moreover, since at least 60% of *Streptomyces* species appear to produce γ-butyrolactones (9), these compounds are likely to play important roles as extracellular signaling molecules in the biology of these organisms. *Streptomyces coelicolor* A3(2), the most genetically characterized streptomycete, produces at least four antibiotics, including the blue-pigmented polyketide actinorhodin (Act),[1] and the red-pigmented tri-pyrolle undecylprodigiosin (Red), both of which are produced in a growth phase-dependent manner (4, 5, 10). Earlier studies (11, 12) led to the isolation and partial structural determination of six γ-butyrolactones made by *S. coelicolor* (Fig. 2), but there was no report of their biological activity in the producing strain.

This paper identifies four low molecular weight compounds present in the supernatants of transition and stationary phase cultures of *S. coelicolor* that can elicit the precocious production of Act and Red when added exogenously to the strain. One of the compounds, SCB1, was purified and its structure ((2*R*,3*R*,1′*R*)-2-(1′-hydroxy-6-methylheptyl)-3-hydroxymethylbutanolide) determined using electron impact mass spectrometry (EI-MS), infrared (IR) spectroscopy, nuclear magnetic resonance (NMR), and circular dichroism (CD).

EXPERIMENTAL PROCEDURES

Strains and Media—*S. coelicolor* M145 (13), and its derivatives M510 (Δ*redD*) and M511 (Δ*act*II-ORF4) (14), were manipulated as described previously (13). SMM (as in Ref. 5, but with no $NH_4(SO_4)_2$ and with phosphate levels reduced to 0.25 mM NaH_2PO_4, 0.25 mM K_2HPO_4), YEME (13), and TSB (13) were used to grow M145 for γ-butyrolactone synthesis, and SMMS (14), MM (13), R2 (13), and R2YE (13) were used to assay for antibiotic production.

Bioassay—Confluent lawns of *S. coelicolor* spores were spread on SMMS plates and allowed to dry. For most assays, 6- or 11-mm diameter holes were made in the agar with a cork-borer, and the bottoms of the holes sealed with 1% agar. Samples in methanol (diluted with an equal volume of 50 mM Tris-HCl, pH 7.2, 1 mM EDTA) or culture supernatants (up to 200 μl) were pipetted into the holes, and the plates incubated at 30 °C for 30–37 h. For some assays, samples were applied directly to the lawn of spores. To determine the minimum concentration of SCB1 required for precocious antibiotic production, the chemically synthesized compound was added to 8 ml of SMMS agar at varying concentrations, and the agar poured into 9-cm diameter Petri dishes. Confluent lawns of M145 spores were added, and the plates incubated at 30 °C for 30–37 h.

Heat and Protease Treatment—Culture supernatants were boiled for 30 min or incubated at 80 °C for 1 h. Protease K, Pronase, and trypsin (all from Sigma) were incubated with culture supernatants at 37 °C for 1 h. The ability of the treated samples to elicit antibiotic production was assayed as described above.

Purification of SCB1—Spores of *S. coelicolor* strain M145 were harvested from a MS (formerly known as SFM; Ref. 14) agar plate and used

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[1] The abbreviations used are: Act, actinorhodin; Red, undecylprodigiosin; EI-MS, electron impact mass spectrometry; IR, infrared; HPLC, high performance liquid chromatography.

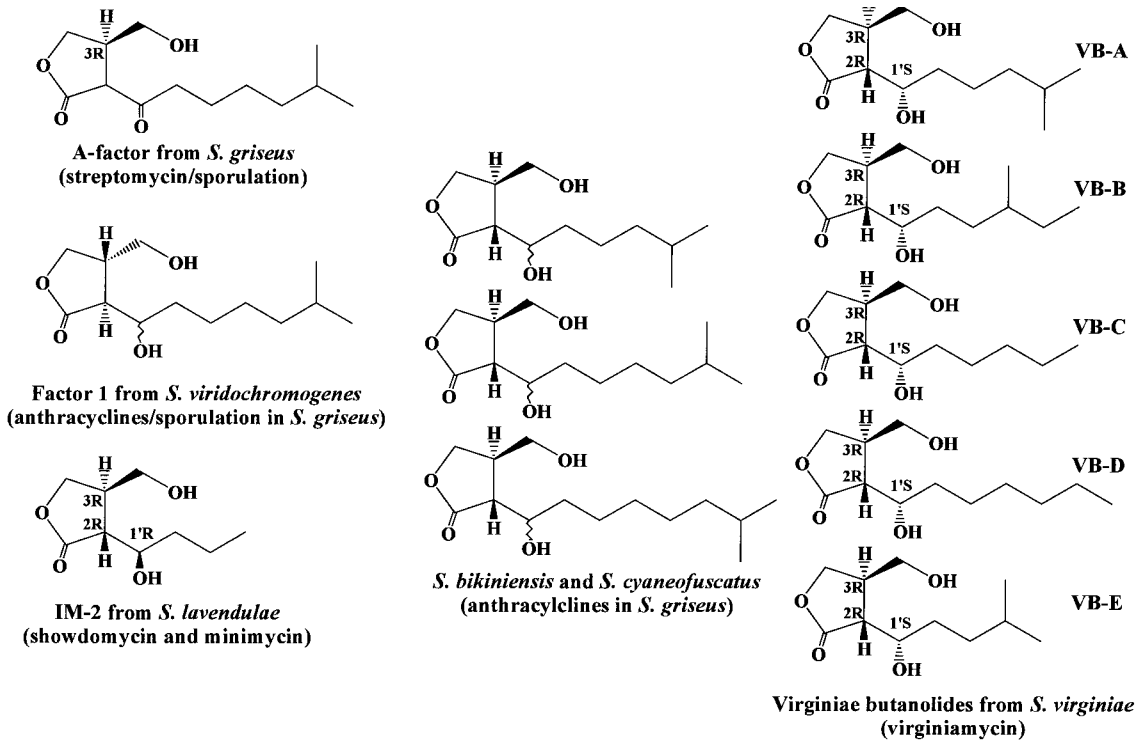

FIG. 1. **γ-Butyrolactones isolated from streptomycetes, and the antibiotics and developmental events induced by them.** The absolute configurations (2*S*,3*S* or 2*R*,3*R*) of Factor I and of the three γ-butyrolactones made by *Streptomyces bikiniensis* and *Streptomyces cyaneofuscatus* were not determined.

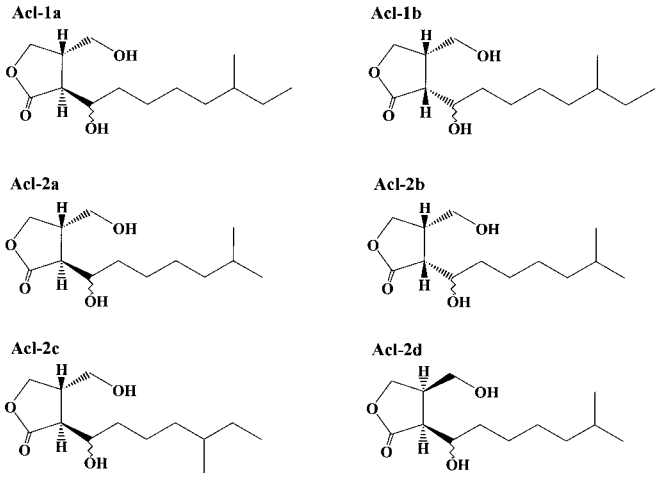

FIG. 2. **Structures of the γ-butyrolactones isolated previously from *S. coelicolor* (from Efremenkova *et al.* (Ref. 12)).**

to generate a three-stage seed culture. Successive seed cultures were grown in shake flasks using a 2% (v/v) inoculum. The cultures were grown at 28 °C and 240 rpm; the primary seed was incubated for 48 h, and the secondary and tertiary seeds for 24 h. The tertiary seed culture was used to inoculate (2% v/v) 300 liters of TSB, which was incubated with pH control at 28 °C for 69 h with an airflow rate of 150 liters min^{-1}, an agitator tip speed of 2 ms^{-1} and an overpressure of 0.5 barg. Approximately 120 g of wet mycelium $liter^{-1}$ were obtained at harvest. The pH of the culture supernatant was adjusted from 8.4 to 7.1 with sulfuric acid, and the resulting 280 liters extracted with 140 liters of ethyl acetate. The extract was dried with $MgSO_4$ and evaporated to give 24.5 g of oil. The oily sample was resuspended in 100 ml of 100% methanol and 20 ml were loaded onto a Silica gel 60 extra pure (Merck) column (bed volume of 230 ml). The sample was eluted with 70% ethyl acetate, 30% n-hexane, yielding fifteen 50-ml fractions. This procedure was repeated five times. Each fraction was subjected to the bioassay. Positive fractions were pooled, filtered through a 0.45-μm cellulose acetate filter (Sartorious), and evaporated to give 495 mg of oily sample. Further purifications were all performed with a C_{18} reverse phase column (4.6 mm × 25 cm Spherisorb 5-μm ODS2, Waters) on a ConstaMetric HPLC equipped with a UV detector (ABI Spectroflow 757) set at 210 nm, and with a flow rate of 1 ml min^{-1}. The oily sample was resuspended in 90% methanol to give a final volume of 10 ml; 200-μl aliquots were loaded onto the column and eluted with a linear gradient of 20% to 100% acetonitrile. Three 7.5-ml fractions were collected and subjected to the bioassay. This procedure was repeated 50 times, and the active fractions were pooled and evaporated to give 190 mg of sample. The material was resuspended in 3 ml of 100% methanol, and 50-μl samples were loaded onto the column and eluted with a linear gradient of 46.3–49.6% acetonitrile. Nine 1-ml fractions were collected and evaporated. This procedure was repeated 23 times. Each fraction was subjected to the bioassay, and the 23 active fractions (one per run) were loaded separately onto the column and eluted with a linear gradient of 46.3–49.6% acetonitrile containing 0.1% trifluoroacetic acid. Peaks were collected manually using a chart recorder (BBC Servogor 120) and subjected to the bioassay. The 23 active peaks (one per run) with the same retention time were pooled and evaporated. The sample was resuspended in 240 μl of 100% methanol, and 20-μl aliquots were loaded onto the column and eluted isocratically with 47% acetonitrile containing 0.1% trifluoroacetic acid. For each of the 12 runs, peaks were collected manually and assayed for bioactivity. Active peaks with the same retention time from each of the 12 runs were pooled, evaporated, and resuspended in 140 μl of 100% methanol. Seven 20-μl aliquots were loaded onto the column and eluted with a linear gradient of 47–47.5% acetonitrile containing 0.1% trifluoroacetic acid. Peaks were collected manually and assayed for bioactivity. Active fractions with the same retention time were pooled, evaporated, and resuspended in 140 μl of 100% methanol. Seven 20-μl aliquots were loaded onto the column and eluted isocratically with 42.5% acetonitrile containing 0.1% trifluoroacetic acid. Peaks were collected manually from each of the seven runs and subjected to the bioassay, and single fractions with bioactivity were evaporated, resuspended in 20 μl of 100% methanol, loaded onto the column, and eluted with a linear gradient of 20–50% acetonitrile containing 0.1% trifluoroacetic acid. Each peak was collected manually and subjected to the bioassay. Active peaks with the same retention time were pooled. Partially purified samples were stored at 4 °C in an evaporated state, and the purified sample was stored at −20 °C in 100% methanol.

Materials for Chemical Synthesis—3-(Hydroxymethyl)butanolide was made by reduction of diethyl formylsuccinate with $NaBH_4$ and converted to 3-(trimethysilyloxymethyl)butanolide by trimethylsilyla-

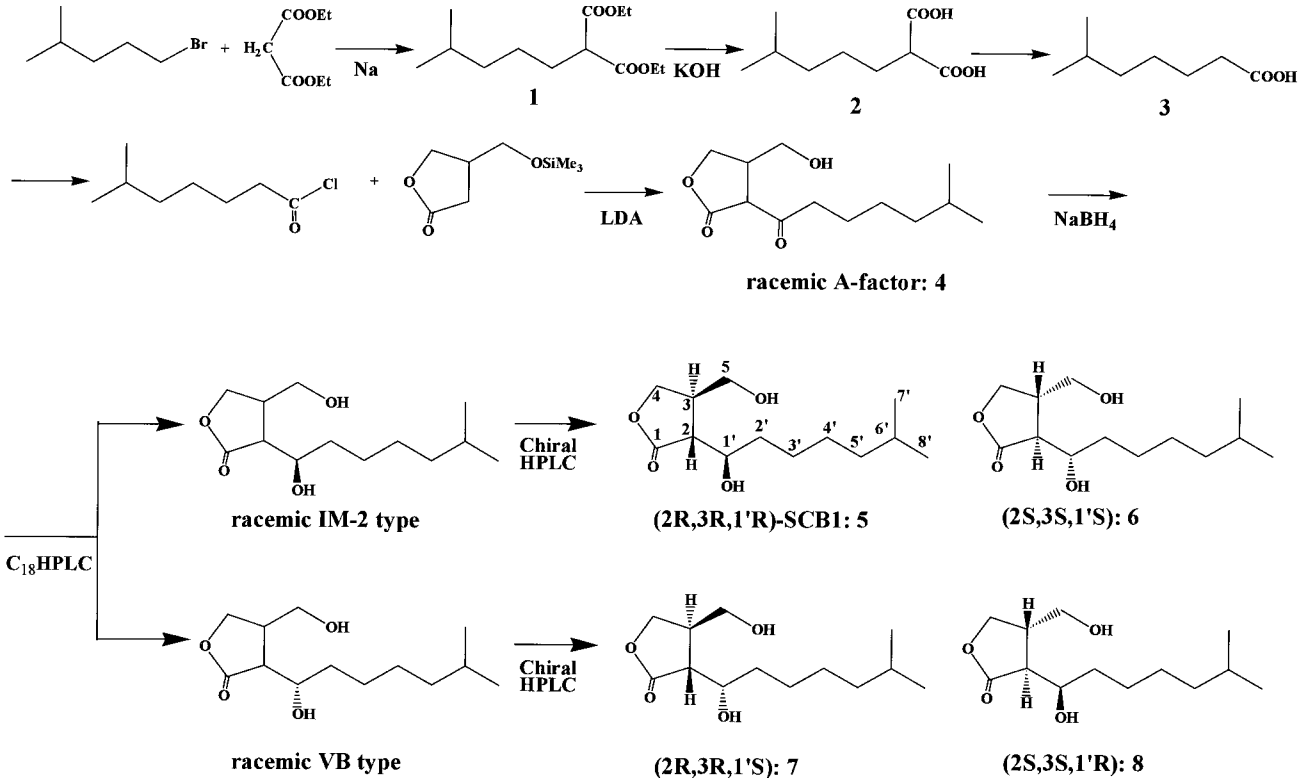

FIG. 3. **Chemical synthesis of SCB1.**

tion (15). 1-Bromo-4-methylpentane was purchased from Aldrich.

Analytical Methods—Infrared (IR) spectra were determined as films on a Horiba model FT-120 Fourier transform-IR spectrophotometer. ^{1}H NMR spectra were recorded on a Varian model Unity Inova 600 spectrometer at 600 MHz using $CHCl_3$ δ_H 7.25 as an internal reference in $CDCl_3$ solution. Optical rotation (CD) values were measured on a Jasco P-1020 polarimeter. Mass spectra were obtained on a Jeol JMS-DX-303 spectrometer.

Physicochemical Data for Natural SCB1—Physicochemical data were as follows: EI-MS m/z 245 $(M+H)^+$, 226 $(M+H\text{-}H_2O)^+$; ^{1}H NMR δ($CDCl_3$, 600 MHz) 4.43 (1H, dd, J = 9 and 8 Hz, H-4a), 4.03 (1H, ddd, J = 9, 5 and 3 Hz, H-1′), 3.99 (1H, dd, J = 9 and 8 Hz, H-4b), 3.77 (1H, dd, J = 10 and 5 Hz, H-5a), 3.70 (1H, dd, J = 10 and 7 Hz, H-5b), 2.79 (1H, m, H-3), 2.66 (1H, dd, J = 9.5 and 5 Hz, H-2), 1.56 (proton numbers ambiguous due to overlapping H_2O signal, m), 1.33 (3H, m, H-4′+6′), 1.19 (1H, t, J = 7 Hz, H-5′a), 1.18 (1H, t, J = 7 Hz, H-5′b), 0.87 (6H, d, J = 7 Hz, H-7′+8′).

Chiral Synthesis of the SCB1 Stereoisomers—For diethyl 2-(4′-methylpentyl)malonate (Fig. 3, *1*), sodium (1.65 g) was added to 40 ml of absolute ethanol, and the mixture stirred at 55 °C until no sodium was visible. After cooling the mixture to <50 °C, diethyl malonate (12.4 g) was added dropwise and refluxed for 20 min. 1-Bromo-4-methylpentane (11.64 g) was added dropwise to the solution with stirring. After refluxing for 2 h, the reaction mixture was evaporated to remove ethanol, acidified with 3 N HCl, and extracted with CH_2Cl_2. The CH_2Cl_2 layer was dried over anhydrous Na_2SO_4, and evaporated to yield 16.1 g of crude 1. For 2-(4′-methylpentyl)malonic acid (Fig. 3, *2*), crude **1** (11 g) was mixed with KOH solution (11 g in 35 ml of water and 80 ml of methanol) and left overnight at room temperature with stirring. The reaction mixture was evaporated to remove methanol, acidified with 3 N HCl, and extracted with CH_2Cl_2. After drying over anhydrous Na_2SO_4, the CH_2Cl_2 layer was evaporated to yield white crystals of 2 (8.2 g, yield 96%). For 6-methylheptanoic acid (Fig. 3, *3*), the white crystals of **2** (8.1 g) were heated at 145 °C in the presence of boiling stones for 8 h. 10% $NaHCO_3$ solution (200 ml) was added, and the mixture extracted once with hexane to remove neutral impurities. The water layer was acidified with 3 N HCl, followed by CH_2Cl_2 extraction. The CH_2Cl_2 layer was dried, evaporated, and the residue was purified by distillation yielding 3.7 g of **3** (yield 60%).

Physicochemical data for **3** are as follows: EI-MS m/z 145 $(M+H)^+$, 127 $(M+H\text{-}H_2O)^+$; ^{1} H NMR δ ($CDCl_3$, 400 MHz) 2.32 (2H, dd, J = 8 and 7 Hz, C$\underline{H}_2$-COOH), 1.59 [2H, dt, J = 15 and 7 Hz, $(CH_3)_2$CH-C$\underline{H}_2$], 1.51 [1H, m, $(CH_3)_2C\underline{H}$], 1.31 (2H, m, -C$\underline{H}_2$-), 1.17 (1H, t, J = 6 Hz, -C$\underline{H}_2$-CH_2-COOH), 1.15 (1H, t, J = 7 Hz, -C$\underline{H}_2$-CH_2-COOH), 0.84 [6H, d, J-7 Hz, $(C\underline{H}_3)_2$-CH]; IR (film) 3037 and 1713 cm^{-1}.

Racemic A-factor (Fig. 3, *4*) (0.7 g, yield 52%) was synthesized by reacting 3-(trimethyl-silyloxymethyl)butanolide (1.03 g) with 6-methylheptanoic acid chloride (0.99 g) in the presence of lithium diisopropylamine in dry tetrahydrofuran under N_2 (15). 6-Methylheptanoic acid chloride was prepared from **3** with oxalyl chloride. Physicochemical data were identical to those reported (16).

Racemic IM-2-type and VB-type SCB1—Racemic A-factor (100 mg in 5 ml of ethanol) was reduced with 1.1 eq of $NaBH_4$ at 4 °C. The reaction was terminated by adding 3 N HCl, and the solution extracted with ethyl acetate. The extract was evaporated, and the residue purified by C_{18} reverse-phase HPLC (Capcell Pak C_{18}, 4.6 × 250-mm column (Shiseido Co. Ltd., Kyoto, Japan), with 35% acetonitrile containing 0.1% trifluoroacetic acid as solvent at a flow rate of 1.5 ml min^{-1} and with detection at 210 nm) to give racemic VB-type SCB1 (retention time 20.1 min, 20% yield) and IM-2-type SCB1 (retention time 22.8 min, 45% yield).

Chiral HPLC—Racemic VB-type SCB1 was separated into two enantiomers by chiral HPLC (Chiralpak AD, 4.6 × 250 mm column (Daicel Co. Ltd., Tokyo, Japan) with hexane:ethanol (92:8) as solvent at a flow rate of 1.1 ml min^{-1}, and with detection at 210 nm) to give (2*R*,3*R*,1′*S*)-SCB1 (retention time 25.5 min, 26% yield) and (2*S*,3*S*,1′*R*)-SCB1 (retention time 15.4 min, 26% yield). Racemic IM-2-type SCB1 was separated similarly (at a flow rate of 0.75 ml min^{-1}) to give (2*R*,3*R*,1′*R*)-SCB1 (retention time 25.1 min, 26% yield) and (2*S*,3*S*,1′*S*)-SCB1 (retention time 28.4 min, 24% yield).

Physicochemical data for synthetic (2*R*,3*R*,1′*R*)-(-)-SCB1 (Fig. 3, *5*) were as follows: $[\alpha]_D^{23} = -20.3°$ (c. 0.065, $CHCl_3$); IR (film) 1755 cm^{-1}; EI-MS m/z 245 $(M+H)^+$, 226 $(M+H\text{-}H_2O)^+$; ^{1}H NMR δ ($CDCl_3$, 600 MHz) 4.43 (1H, dd, J = 9 and 8 Hz, H-4a), 4.03 (1H, ddd, J = 9, 5 and 3 Hz, H-1′), 3.99 (1H, dd, J = 9 and 8 Hz, H-4b), 3.77 (1H, dd, J = 10 and 5 Hz, H-5a), 3.70 (1H, dd, J = 10 and 7 Hz, H-5b), 2.79 (1H, m, H-3), 2.66 (1H, dd, J = 9.5 and 5 Hz, H-2), 1.56 (4H, m, H-2′+3′), 1.34 (3H, m, H-4′+6′), 1.19 (1H, t, J = 7 Hz, H-5′a), 1.18 (1H, t, J = 7 Hz, H-5′b), 0.87 (6H, d, J = 7 Hz, H-7′+8′).

Physicochemical data for synthetic (2*S*,3*S*,1′*S*)-(+)-SCB1 (Fig. 3, *6*) were as follows: $[\alpha]_D^{23} = +16.5°$ (c. 0.06, $CHCl_3$); ^{1}H NMR, EI-MS, and IR data were identical to those for (2*R*,3*R*,1′*R*)-(−)-SCB1.

Physicochemical data for synthetic (2*R*,3*R*,1′*S*)-SCB1 (Fig. 3, *7*): $[\alpha]_D^{23}$= -32.8° (c. 0.065, $CHCl_3$); EI-MS m/z 245 $(M+H)^+$, 226 $(M+H\text{-}H_2O)^+$; IR (film) 1749 cm^{-1}; ^{1}H NMR δ ($CDCl_3$, 600 MHz) 4.40 (1H, dd, J = 9 and 8 Hz, H-4a), 4.11 (1H, m, H-1′), 4.08 (1H, dd, J = 9 and 8 Hz,

H-4b), 3.74 (1H, dd, J = 10 and 5 Hz, H-5a), 3.70 (1H, dd, J = 10 and 6 Hz, H-5b), 2.84 (1H, m, H-3), 2.55 (1H, dd, J = 7.1 and 4 Hz, H-2), 1.53 (4H, m, H-2′+3′), 1.28 (3H, m, H-4′+6′), 1.16 (2H, ddt, J = 14, 7 and 2 Hz, H-5′), 0.85 (6H, d, J = 7 Hz, H-7′+8′).

Physicochemical data for synthetic (2*S*,3*S*,1′*R*)-SCB1 (Fig. 3, *8*) were as follows: $[\alpha]_D^{23}$ = +28.2° (c. 0.065, $CHCl_3$); ^{1}H NMR, EI-MS, and IR data were identical to those for (2*R*,3*R*,1′*S*)-SCB1.

RESULTS

Transition and Stationary Phase Culture Supernatants of S. coelicolor Elicit Precocious Antibiotic Production—Exponential phase (A_{450} = 0.4), late exponential phase (A_{450} = 0.7), early transition phase (A_{450} = 1.0), late transition phase (A_{450} = 1.5), and stationary phase (A_{450} = 2.0) culture supernatants of *S. coelicolor* M145 grown in SMM were assayed for their ability to elicit precocious production of the pigmented antibiotics Act and Red in lawns of the same strain grown on SMMS. While supernatants from exponential and early transition phase cultures showed no activity, those from late transition phase and stationary phase cultures appeared to elicit production of both antibiotics (Fig. 4*A*). Exponential phase culture supernatants concentrated 50-fold by freeze-drying failed to show activity. Essentially the same results were obtained when M145 was grown in complex YEME and TSB liquid media, and when MM, R2, and R2YE rather than SMMS were used to assay for precocious antibiotic production. The ability of the culture supernatants to cause premature Act and Red production on several defined and complex media suggests that the activity of the factor(s) responsible is not highly dependent on the nutritional state of the culture, and is consistent with a key role for the factor(s) in determining the onset of antibiotic production. To establish unambiguously that the observed pigment production reflected synthesis of both of the colored antibiotics, mutants carrying deletions in one or other of the pathway-specific activator genes for Act (*act*II-ORF4) and Red (*redD*) synthesis, M511 and M510, respectively, were used as lawns in the bioassay. The late transition and stationary phase culture supernatants stimulated the production of Act in M510 and Red in M511 (Fig. 4*B*), confirming the induced synthesis of both of the pigmented antibiotics. Interestingly, the stimulatory effect was only observed when the culture supernatants were added within 8 h of spreading the lawns of M145, M510, or M511 spores; later addition had no effect.

Stimulation of Antibiotic Production Is Caused by Small Diffusible Factors—When a stationary phase culture supernatant of M145 that possessed stimulatory activity was dialyzed against 50 mM Tris-HCl, pH 7.2, 1 mM EDTA, all activity was lost. Use of a Centricon-3 (Amicon) filter indicated that the molecular weight of the active factor(s) was less than 3000. The stimulatory activity was resistant to protease and heat treatment (100 °C for 30 min and 80 °C for 1 h); it was stable in acidic conditions (pH1.0 for 30 min), unstable in alkali (pH12.0 for 10 min), and was readily extracted into ethyl acetate and chloroform. Moreover, C_{18} reverse phase HPLC using an acetonitrile/water mobile phase gave a peak with bioactivity that eluted at approximately 50% acetonitrile. All of these properties are characteristics associated with γ-butyrolactones.

Purification of the Major Stimulatory Factor Made by S. coelicolor—A 300-liter culture of *S. coelicolor* M145 was grown in TSB at 28 °C for 69 h, and the supernatant extracted with ethyl acetate at neutral pH. After evaporation, the oily extract was passed through a silica gel column using 70% ethyl acetate, 30% *n*-hexane as solvent, and fractions with stimulatory activity were obtained. Further purification was achieved by C_{18} reverse phase HPLC using an acetonitrile gradient as mobile phase. To maximize the separation of individual peaks, an analytical column was used throughout the remainder of the purification. Two rounds of C_{18} reverse phase HPLC yielded two peaks with bioactivity. The major peak eluted at 47.0% acetonitrile, and the minor peak at 48.2%. The minor peak was later shown to contain at least two stimulatory activities. Further purification was performed only with the major peak. Six additional rounds of C_{18} reverse phase HPLC gave a single peak with stimulatory activity, which was designated SCB1. A small peak with stimulatory activity that had a retention time just slightly longer than that of SCB1 (10.8 min *versus* 9.9 min on 42.5% acetonitrile isocratic elution) was also identified, but there was too little material for structural determination.

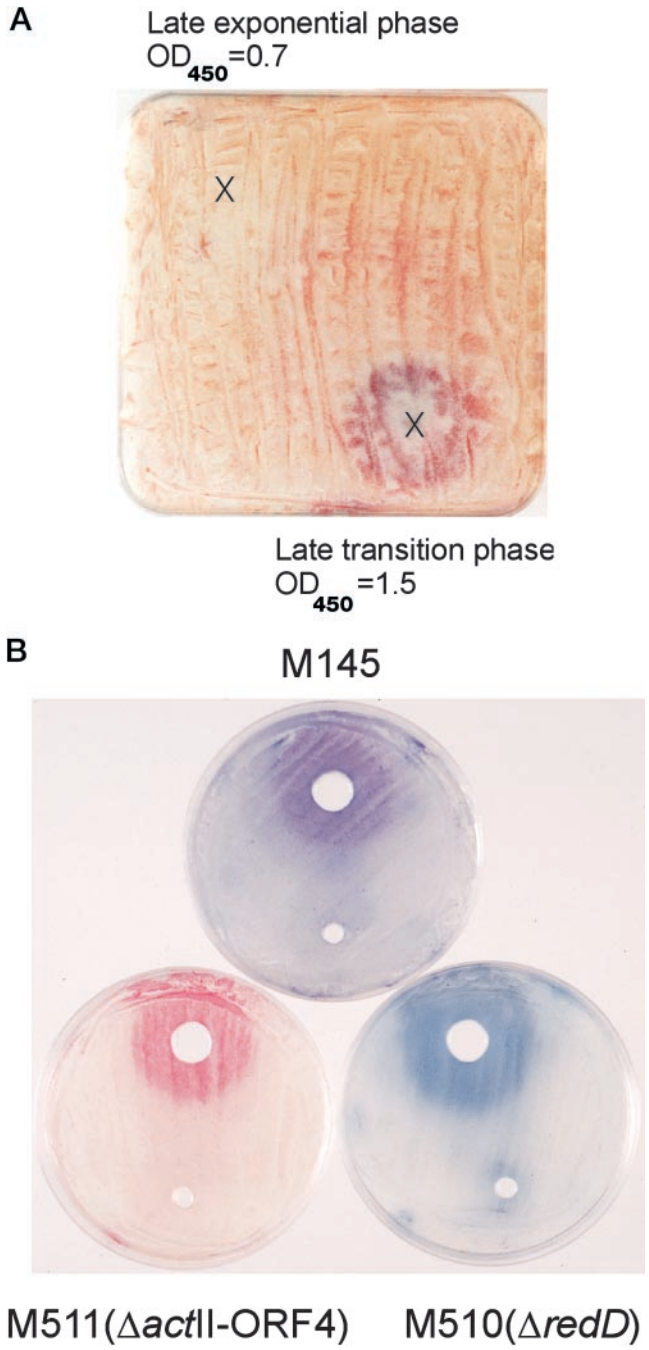

FIG. 4. **Bioassay of culture supernatants of *S. coelicolor* M145.** *A*, ethyl acetate extracts (1 μl) of supernatants of late exponential and late transition phase cultures were spotted (*X*) on a SMMS plate seeded with a confluent lawn of M145 spores. *B*, stationary phase culture supernatants were placed in wells (200 μl in the top wells and 50 μl in the bottom wells) made in an agar plate that had been spread with a confluent lawn of spores of *S. coelicolor* M145 (*top*), M511 (Δ*act*II-ORF4) (*bottom left*), and M510 (Δ*redD)* (*bottom right*). Plates were incubated at 30 °C for 30–37 h.

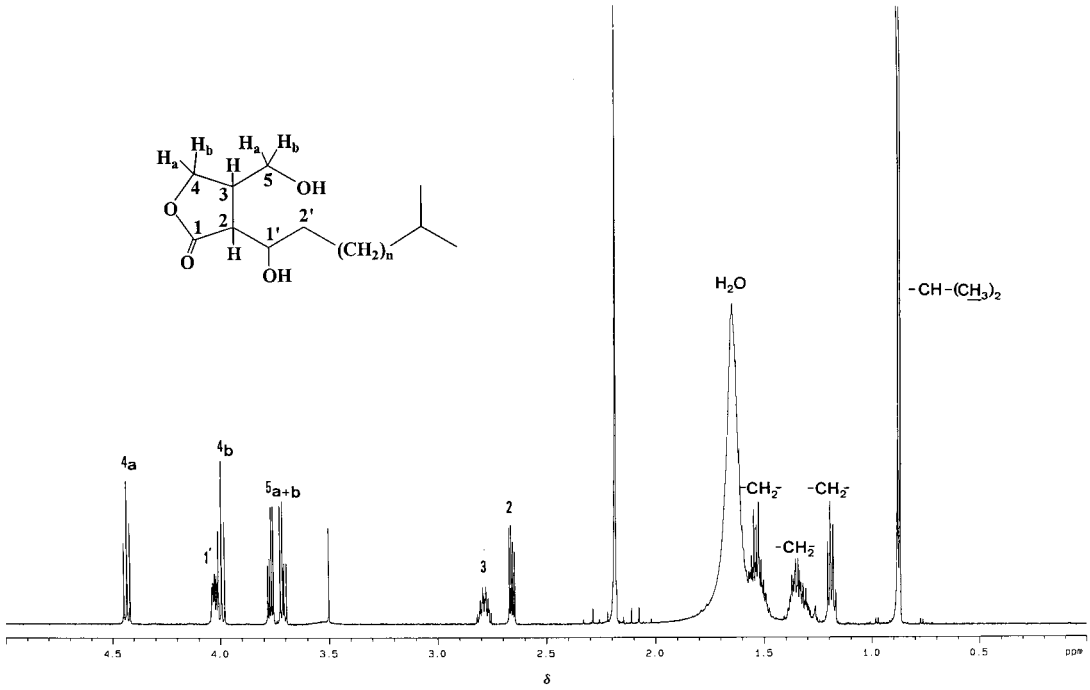

FIG. 5. **600-MHz ^{1}H NMR spectrum of SCB1.** The predicted plain structure of SCB1 is shown in the *inset*. The *number* above each signal indicates the corresponding proton in SCB1.

Thus, *S. coelicolor* produces at least four compounds that can induce precocious antibiotic production.

Structure of SCB1—One-dimensional 600-MHz ^{1}H NMR (Fig. 5) indicated that the purified compound possessed a typical 2,3-disubstituted γ-butyrolactone skeleton, as evident from the signals at 4.43, 4.03, 3.99, 3.77, 3.70, 2.79, and 2.66 ppm. The signals at 4.43 and 3.99 ppm correspond to methylene protons at C-4 (Fig. 3, *5*), and those at 3.77 and 3.70 ppm to hydroxymethylene protons at C-5. The signals at 4.03, 2.79, and 2.66 ppm correspond to three methine protons at C-1′, C-3, and C-2, respectively. The remaining signals at 1.56, 1.33 (3 protons), 1.19 (2 protons), and 0.87 ppm (6 protons) appeared to originate from an alkyl side chain at C-2, although the broad signal of contaminating H_2O at 1.7 ppm overlapped the 1.56 ppm signal, preventing determination of the number of protons in the 1.56 ppm signal. However, the doublet signal at 0.87 ppm corresponds to six protons of two methyl groups and indicated the presence of a terminal isopropyl moiety. Supposing that two to four methylene protons in the 1.56 ppm signal were masked by the 1.7 ppm H_2O signal, the structure of the C-2 side chain could be either 1′-hydroxy-5′-methylhexyl (n = 2) or 1′-hydroxy-6′-methylheptyl (n = 3). To confirm these predictions, a two-dimensional H-H COSY spectrum of SCB1 was determined (Fig. 6). All of the expected couplings were observed.

$$\begin{array}{l} \text{H-4}_b\ (3.99\ \text{ppm}) \rightarrow \text{H-4}_a\ (4.43) \\ \qquad\qquad \downarrow \\ \qquad \textit{H}\text{-3}\ (2.79\ \text{ppm}) \rightarrow \textit{H}\text{-5}_{a+b}\ (3.77\ \textit{and}\ 3.70\ \text{ppm}) \\ \qquad\qquad \downarrow \\ \qquad\qquad \text{H-2}\ (2.66\ \text{ppm}) \rightarrow \text{H-1}'\ (4.03\ \text{ppm}) \rightarrow \text{H-2}'\ (1.56\ \text{ppm}) \end{array}$$

SCHEME 1

Several attempts to eliminate contaminating water from the sample were unsuccessful, reflecting the limiting amount of purified compound (~320 μg) available; this prevented determination of the number of methylenes between C-1′ and the terminal isopropyl moiety by ^{1}H NMR. However, EI-MS of the purified compound gave a clear parent signal of m/z 245 $(M+H)^+$, indicating that SCB1 has a 1′-hydroxy-6′-methylheptyl group at C-2, and a molecular weight of 244.

The coupling constant between H-2 and H-3 ($J_{2,3}$=9.5 Hz) was different from that of VB-A ($J_{2,3}$=7.4 Hz) (17), but agreed well with that of IM-2 ($J_{2,3}$=9.3 Hz) (18), suggesting that SCB1 possesses an IM-2-type stereochemistry about C-2, 3 and 1′. To confirm the IM-2-type nature of SCB1, the retention time of the purified compound upon C_{18} reverse phase HPLC was compared with those of synthetic IM-2-type and VB-type γ-butyrolactones. Samples were prepared by $NaBH_4$ reduction of synthetic A-factor followed by purification on reverse-phase HPLC. Natural SCB1 and the IM-2-type reduced A-factor derivative eluted at 11.8 min, while the VB-type reduced A-factor eluted at 10.2 min, indicating that natural SCB1 has an IM-2-like stereochemistry, either 2*R*,3*R*,1′*R* or the enantiomeric 2*S*,3*S*,1′*S*.

Chemical Synthesis of SCB1 and Its Stereoisomers—To determine the absolute configuration of SCB1, the IM-2-type enantiomers (2*R*,3*R*,1′*R* and 2*S*,3*S*,1′*S*) and the VB-type enantiomers (2*R*,3*R*,1′*S* and 2*S*,3*S*,1′*R*) of SCB1 were chemically synthesized (Fig. 3). Racemic A-factor was synthesized by acylating 3-(trimethylsilyloxymethyl)butanolide with 6-methylheptanoic acid chloride. Reduction with $NaBH_4$ and separation by C_{18} reverse-phase HPLC gave racemic mixtures of the IM-2-type and VB-type compounds. Separation of each racemic mixture by chiral HPLC yielded optically pure compounds. Since natural VB-A and IM-2 possess negative optical rotations (CD-) and their absolute configurations had been determined unambiguously to be 2*R*,3*R* (19), each of the chemically synthesized stereoisomers that possessed a negative optical rotation was assigned as 2*R*,3*R*. The elution time of natural SCB1 from a Chiralpak AD column using hexane:ethanol (92:8) as mobile phase at a flow rate of 0.5 ml min^{-1} was 33.3 min, identical to that of the synthetic 2*R*,3*R*,1′*R* isomer, whereas the 2*S*,3*S*,1′*S* isomer eluted after 30.4 min. Consequently, the structure of natural SCB1 was deduced to be (2*R*,3*R*,1′*R*)-2-(1′-hydroxy-6-methylheptyl)-3-hydroxymethylbutanolide (Fig. 3).

Minimum Concentration of SCB1, Its Stereoisomers, and Other γ-Butyrolactones Required to Elicit Precocious Antibiotic

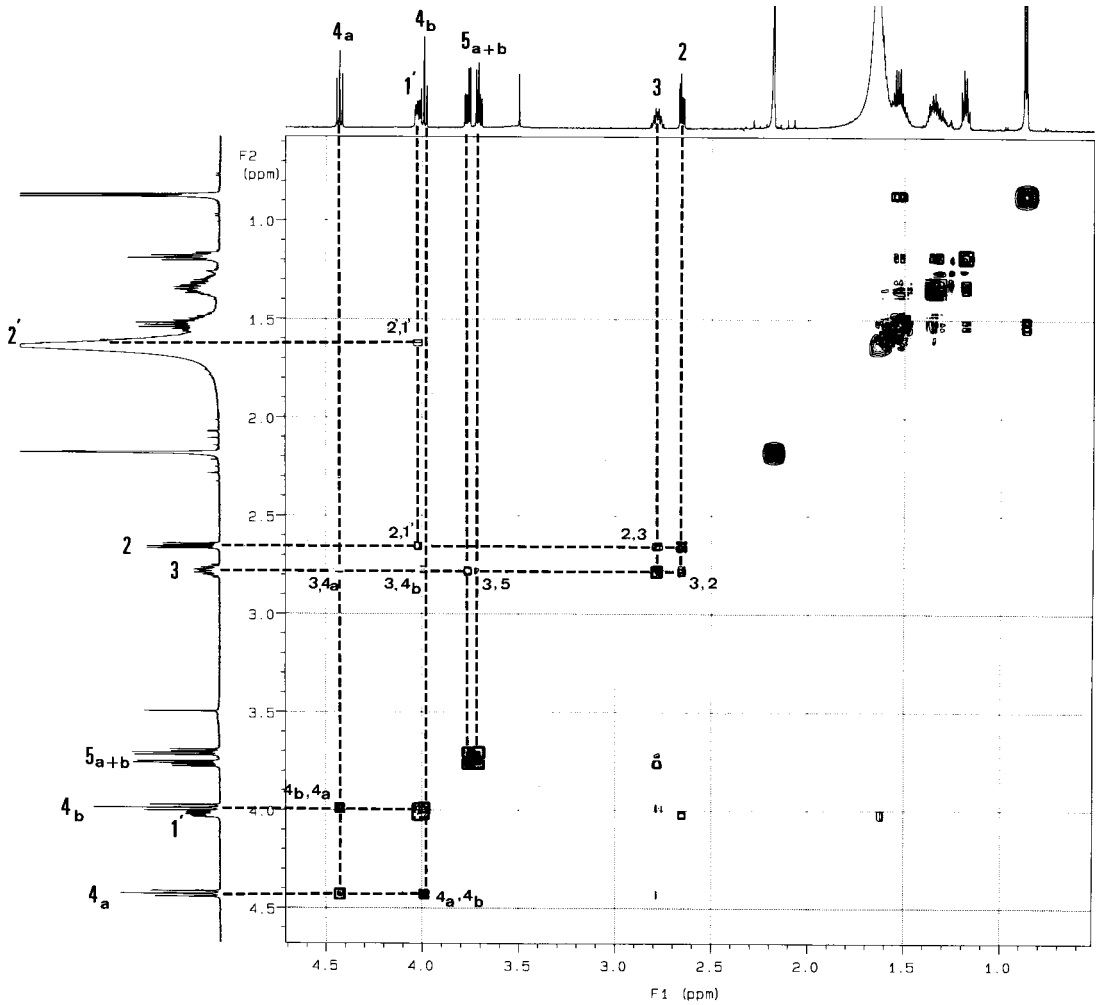

FIG. 6. **Two-dimensional H-H COSY spectrum of SCB1.** The *numbers* adjacent to the signals indicate the cross-peaks between the corresponding protons in SCB1 (see Fig. 5).

Production in S. coelicolor—The minimum concentration of each of the four stereoisomers of SCB1 needed to induce antibiotic production in the SMMS plate bioassay was determined to be: SCB1 (2*R*,3*R*,1′*R*), between 128 and 256 nM (Fig. 7); (2*S*,3*S*,1′*S*), 410 and 4100 nM; (2*R*,3*R*,1′*S*), 410 and 4100 nM; (2*S*,3*S*,1′*R*), 780 and 7800 nM. The concentration of SCB1 required to elicit antibiotic production in *S. coelicolor* appears to be considerably higher than the levels of A-factor (about 1 nM; Ref. 20) and virginiae butanolides (about 3 nM; Ref. 21) required to induce antibiotic production in *S. griseus* and *S. virginiae*, respectively, but corresponds to the level of stimulatory activity observed on bioassay of transition and stationary phase supernatants of SMM-grown cultures (the zones of antibiotic production indicated stimulatory activity equivalent to approximately 250 nM SCB1). Interestingly, high concentrations of SCB1 (>1,000 nM) inhibited Act and Red production in *S. coelicolor* (Fig. 7), but had no inhibitory effect on growth or morphological differentiation. An inhibitory effect was also observed for high concentrations (>1, 400 nM) of racemic VB-C on virginiamycin production in *S. virginiae*,[2] and for high concentrations of A-factor (410–4100 nM) on the formation of aerial mycelium in *S. griseus* (Ref. 11; the effect on streptomycin production was not reported). Both A-factor and a C_5 derivative of IM-2 elicited precocious antibiotic production in *S. coelicolor* at concentrations of 26,000 and 495,000 nM, respectively, far higher than those required for SCB1 (<256 nM), while VB-C was inactive at a concentration of 440,000 nM.

DISCUSSION

A γ-butyrolactone, SCB1, with the ability to induce the precocious production of both Act and Red in *S. coelicolor,* was purified to homogeneity and its structure determined. SCB1 does not correspond to any of the six γ-butyrolactones reported to be made by *S. coelicolor* (12) (Fig. 2). The biological activity of these compounds in *S. coelicolor* was not described, although they restored streptomycin production and sporulation to an A-factor-deficient mutant of *S. griseus*, albeit at a 500-fold higher concentration than A-factor itself. Whether SCB1 also has this activity remains to be determined. SCB1 may be identical to factor I, a γ-butyrolactone made by *Streptomyces viridochromogenes* (22) (Fig. 1); while the C2, C3 positions of Factor I were defined as *trans* (*i.e.* 2*S*,3*S* or 2*R*,3*R*), and the configuration of the hydroxyl at C1′ as β (23), as in SCB1, the absolute configuration of the molecule was not determined. While this compound was able to induce leucomycin production and aerial mycelium formation in *S. griseus* ZIMET 43682, its role in the producing organism was not reported. Recently, γ-butyrolactone-like compounds were identified in *S. coelicolor* that were able to elicit precocious morphological differentiation (21) but not antibiotic production[2] in the producing strain, the converse of the phenotypes associated with SCB1. It thus appears that *S. coelicolor* produces several different γ-butyrolac-

[2] T. Nihira, unpublished data.

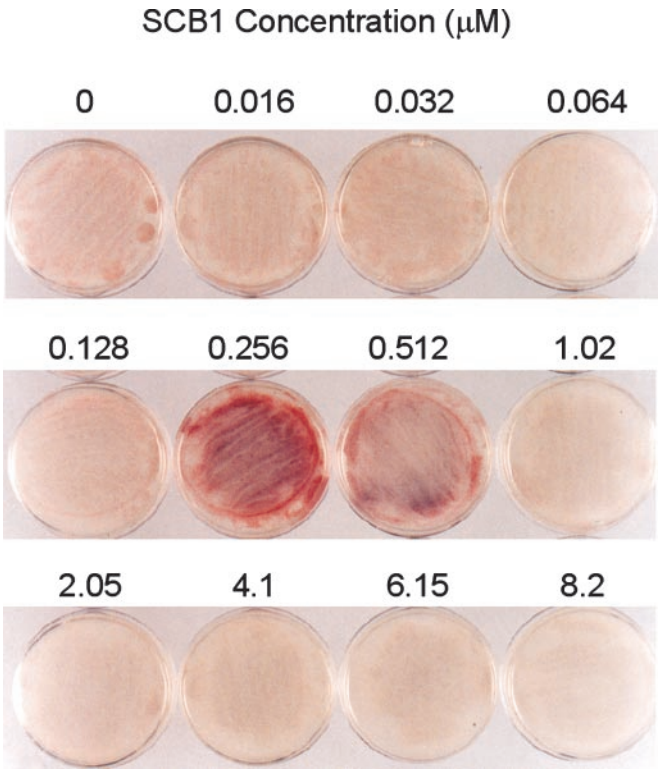

FIG. 7. **Concentration of purified natural SCB1 required for precocious antibiotic production.** Plates were incubated at 30 °C for 37 h.

tones with distinct biological activities.

Each of the three chemically synthesized stereoisomers possess markedly weaker activities than SCB1, indicating that the C2, C3, and C1′ positions play important roles in determining the specificity of the γ-butyrolactone for its presumed binding protein. A-factor and IM-2-C_5 could both elicit antibiotic production in *S. coelicolor*, but at much higher concentrations than SCB1. The carbonyl group at the C1′ position of A-factor is susceptible to reduction; consequently, a low level of SCB1 present in the A-factor sample may be responsible for the relatively high apparent stimulatory activity of A-factor compared with that of IM-2-C_5 and VB-C.

In addition to SCB1, three other stimulatory activities were observed in culture supernatants of *S. coelicolor*. Two of these eluted markedly later than SCB1 from the C_{18} column, and are likely to be γ-butyrolactones with longer carbon side chains than SCB1. While they may correspond to Acl-1a and Acl-1b (12) (Fig. 2), since all of the streptomycete γ-butyrolactones whose structures have been absolutely determined possess the 2*R*,3*R* configuration, it seems more likely that they correspond to the 2*R*,3*R* diastereomers of these compounds. Interestingly, the stimulatory effect elicited by addition of culture supernatants containing these compounds to lawns of *S. coelicolor* spores was only observed if the supernatants were applied within 8 h of plating out the lawns; later addition had no stimulatory effect. This is reminiscent of the "decision phase" described for the effective addition of A-factor to a culture of *S. griseus* deficient in A-factor production (12, 24); restoration of streptomycin production was only observed if A-factor was added within 10 h of inoculation. It will be interesting to determine the molecular basis for this apparent growth phase-dependent response to γ-butyrolactone addition.

In *S. griseus,* only one γ-butyrolactone, A-factor, appears to regulate streptomycin production, and in *Streptomyces lavendulae*, a single γ-butyrolactone, IM-2, induces production of the nucleoside antibiotics showdomycin and minimycin (25). In contrast, *S. virginiae* produces at least five virginiae butanolides (VB-A, B, C, D, and E) that stimulate virginiamycin production, each with a different minimum effective concentration (26). Like *S. virginiae, S. coelicolor* appears to produce several γ-butyrolactones that are capable of stimulating the production of at least two chemically distinct antibiotics. It will be interesting to determine how these γ-butyrolactones elicit their pleiotropic effect on antibiotic production.

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